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SLOW MONO- AND BIPHASIC Ca^{2+}-BINDING KINETICS TO Ni^{2+}-CONCANAVALIN A.

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SUMMARY : Biphasic kinetics of Ca^{2+} binding to Ni^{2+}-concanavalin A were studied at pH 5.2 and 16 °C in the min-time range, using 4-methylumbelliferyl α-D-mannopyranoside as an indicator. The results contrast with the monophasic Ca^{2+}-induced decrease of Tyr absorption followed in the same time range. Both kinetic phenomena can be consistent with two protomeric forms of dimeric Ni^{2+}-concanavalin A in solution.

Binding of metal ions at the binuclear site in apo-concanavalin A is required prior to the binding of carbohydrates by dimeric concanavalin A (con A)[1] [see refs 10-37 in (1)]. The specific binding of Ca^{2+} to transition-metal-ion con A can be monitored with carbohydrates (2-5) without influence of Ca^{2+} binding at secondary sites (6-7). Here we mainly use the total fluorescence quenching of 4-methylumbelliferyl α-D-mannopyranoside (S) upon its relatively fast and well characterized binding reaction (4,5,8,9) with the fully metallized lectin. These results indicate that the kinetics of Tyr-absorption decrease (10) describe only a part of the specific Ca^{2+}-binding process.

MATERIALS AND METHODS

All solutions were made up in doubly distilled water and "Suprapur" (Merck) chemicals were used. A con A preparation (11), treated with NH_4HCO_3, (12) and essentially devoid of nicked polypeptide chains was demetallized (4 °C; pH 1.2) and dialyzed (6). Any remaining native protein was adsorbed

[1] Abbreviations : con A, concanavalin A ; S, 4-methylumbelliferyl α-D-mannopyranoside.

[2] This was observed for binding of Ca^{2+} (0.5 to 80 mM) to aged or to freshly prepared Ni^{2+}-con A. These slow monophasic kinetics, together with the process faster than 5 sec, agree with the data in a recent report (16) on Ca^{2+} binding to Mn^{2+}-con A. For a single Ca^{2+} concentration (0.3 mM), binding to Mn^{2+}-con A was reported as biphasic in the min-time range (16). In our experiments, the slow monophasic binding of Ca^{2+} even as low as 0.5 mM was never affected by the process faster than 5 sec.

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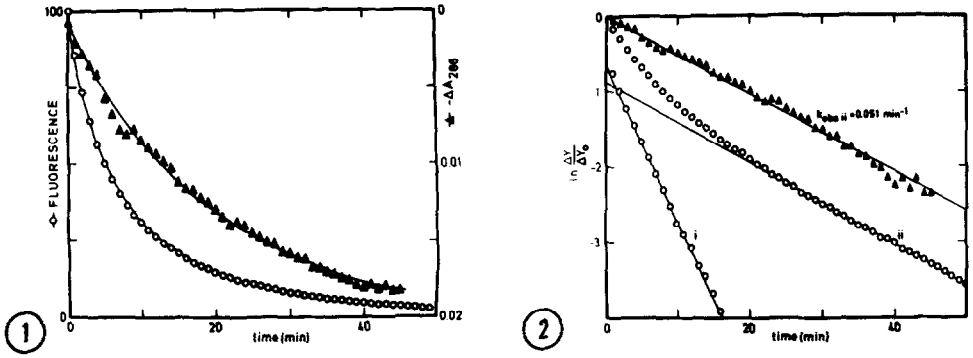

Fig. 1. Comparison of the Ca^{2+} binding kinetics to Ni^{2+}-con A observed by Tyr absorption and by S-fluorescence quenching. The monophasic absorption decrease (▲) was obtained with 1.6 mM Ca^{2+} and 60 µM Ni^{2+}-con A and the smooth curve corresponds to $-\Delta A = 0.95 \exp(-0.051\ min^{-1} xt)$. A fast process, corresponding to 5% of the signal change was complete in less than 5 sec. The biphasic S-fluorescence decrease (o) was obtained with 1.3 mM Ca^{2+}, 60 µM Ni^{2+}-con A and 2 µM S and no fast process was observed. The total absorbance change ($-\Delta A = 0.020$) and the effective fluorescence quenching (70%) are represented on the same arbitrary scale (0-100). It corresponds to Fo-Fe were Fo is the starting fluorescence and Fe is the fluorescence at equilibrium measured at 120 min. The smooth curve is computed with $F = \phi S = \phi\{So - 0.5[X - (X^2 - 4ASo)^{0.5}]\}$ in which ϕ is a calibration factor, So = 2 µM, [S] is the concentration of S at any time and X is the sum of the three parameters So, the dissociation constant of S (B) and the concentration of fully metallized con A at any time; this value, A, equals $[Fo(F-Fe)]/[F(Fo-Fe)] = A_i \exp(-k_{obsi} xt) + A_{ii} \exp(-k_{obsii} xt)$ in which F is the fluorescence at any time, k_{obsi} and k_{obsii} are the rate parameters for the fast and slow processes i and ii. A_i and A_{ii} are the fractions of A formed in these processes. These parameters are obtained from the corresponding linearized expression.

Fig. 2. Comparison of the linearized relative signal changes of Tyr absorption and S fluorescence from Fig. 1. The logarithm of the relative signal change $\Delta Y/\Delta Y_o$ is linear when measured in absorption ($\Delta A/\Delta A_o$, ▲) and corresponds to a reaction rate constant of 0.051 min^{-1}. When measured in fluorescence [o, (F-Fe)/Fo-Fe)] the process contains a fast (i) and a slow process (ii). The rate constants describing the change in fluorescence in terms of the fractional protein contribution were obtained (15) by linearizing the expression given with Fig. 1 and correspond to $k_{obsi} = 0.161\ min^{-1}$ and $k_{obsii} = 0.049\ min^{-1}$.

on a column of Sephadex G-75. The eluted apo-con A contained 0.006 gramatom Mn^{2+} and 0.025 gramatom Ca^{2+}/25,500 D and was stored at -15 °C. Ni^{2+}-con A was prepared by adding 2.5 equivalents of $NiCl_2$ to 0.4 mM apo-con A and standing at 25 °C for 8 days prior to use (13). All con A concentrations were determined at 280 nm using $\varepsilon = 2.91 \times 10^4\ M^{-1}\ cm^{-1}$ for the protomer.

All experiments were performed with 58 to 66 µM dimeric Ni^{2+}-con A at (16 ±0.2)°C and pH 5.2 (6). Pseudo-first-order kinetics of Ca^{2+} binding were initiated by addition of a concentrated $CaCl_2$ solution in two types of experiments and were followed for 30 min: a) differential Tyr-absorption decrease at 286 or 294 nm was recorded in the absence of carbohydrate on a 0.020 absorbance scale with a Zeiss PM QII spectrophotometer, b) S-fluorescence quenching (2 µM, excitation at 313 nm, emission above 360 nm), indicating Ca^{2+} binding to Ni^{2+}-con A, was recorded with a Vitatron

photometric system (8); the inner-filter effect (A = 0.026) and photolysis (0.3% or less) were negligible. The total signal change was determined after 60 min (a) or 120 min (b). In some experiments fluorescence of 250 µM S was excited at 358 nm in an Aminco SPF 500 instrument.

RESULTS

Binding of an excess of [Ca^{2+}] with Ni^{2+}-con A at 16 °C, as monitored by the decrease in fluorescence of 2 µM S deviates from a simple first order reaction and shows two kinetic processes i and ii in the min-time range (Fig. 1, Fig. 2). This biphasic character could also be observed by fluorescence of 250 µM S and by the difference absorption of 150 µM S (334 nm) and of p-nitrophenyl α-D-mannopyranoside at 315 nm (14). In all experiments, [Ca^{2+}] was saturating as judged from the constant total signal change with (70 ±7)% effective quenching. The binding of Ca^{2+} to Ni^{2+}-con A as observed through S fluorescence quenching, is characterized by two rate constants, $k_{obs,i}$ and $k_{obs,ii}$. These were determined (15) according to Fig. 1, differ by a factor 3 to 4 and increase with [Ca^{2+}] to approach constant values (Fig. 3).

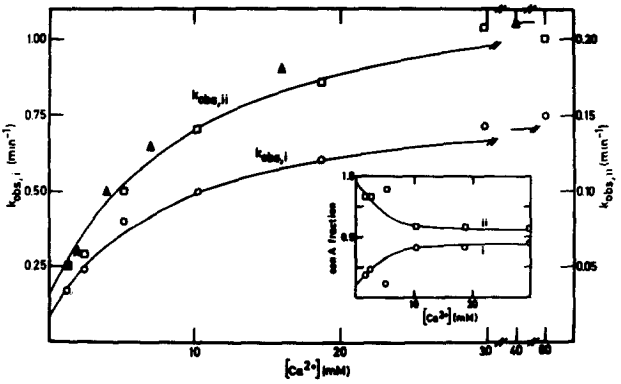

Fig. 3. Ca^{2+} dependence of the rate constants $k_{obs,i}$ and $k_{obs,ii}$, calculated from the biphasic S-fluorescence quenching (o $k_{obs,i}$, □ $k_{obs,ii}$; Fig. 1, legend) and from the monophasic Tyr-absorption decrease (▲ $k_{obs,ii}$, Fig. 2). For processes i and ii the rates correspond to scheme 1 with $k_{obs} = k_{-b} + (k_{+b} + K_a[Ca^{2+}])/(1 + K_a[Ca^{2+}])$; these curves are simulated with k_b = 0.75 M^{-1} min^{-1}, k_{-b} = 0.08 min^{-1} and K_a = 120 M^{-1} for $k_{obs,i}$ and with k_b = 0.22 M^{-1} min^{-1}, k_{-b} = 0.03 min^{-1} and K_a = 100 M^{-1} for $k_{obs,ii}$. The inset shows the [Ca^{2+}] dependence of the fractional protein contributions (o A_i, □ A_{ii}; see Fig. 1, legend), suggesting scheme 2.

The fractions of metallized con A, reacting through processes i and ii are A_i and A_{ii} (Fig. 3, legend). A_i increases with $[Ca^{2+}]$ from 0.1-0.2 to approach 0.5; concomittantly, A_{ii} decreases from 0.9-0.8 to 0.5 (Fig. 3, inset). The latter observations exclude two independent Ca^{2+}-binding reactions of Ni^{2+}-con A.

In similar experiments, but in the absence of any carbohydrate, the observed decrease of Ca^{2+}-induced Tyr absorption is monophasic[2] in the min-time range (Fig. 1). This slow and monophasic decrease in absorption seems identical to the slower process ii; the $k_{obs,ii}$ values are comparable when determined by either method (Fig. 3). The trace of the Ca^{2+}-induced decrease of Tyr absorption is preceded by a very fast process which is complete in less than 5 sec. The fractional contribution of this very fast process to the total signal change increases with $[Ca^{2+}]$ (1-80 mM) from 0.05 (Fig. 1) to 0.35 and the fractional contribution of the slow process ii decreases from 0.95 to 0.65.

DISCUSSION

Upon binding of Ca^{2+} to Ni^{2+}-con A at 16 °C, the number of kinetic processes observed in the min-time range depends on the origin of the optical signal change. The latter is monophasic for protein absorption and biphasic for fluorescence of e.g. 2 µM S. This difference between mono- and biphasic kinetics is already apparent at low $[Ca^{2+}]$ (Fig. 1, Fig. 2) and becomes even more pronounced at higher $[Ca^{2+}]$ (not shown). It was found that the faster process i, observed e.g. through fluorescence of S is also observed through difference absorption of S or p-nitrophenyl α-D-mannopyranoside. Furthermore, process i is by far too large to be an artifact caused by binding of S with $t_{0.5} < 0.5$ sec (9,17). As shown by simulation, the latter reaction would cause a negligible error (< 0.05‰; 0.01‰ at 1; 3 min) when it indicates a mono-exponential activation of Ni^{2+}-con A with e.g. $k = 0{,}2\ M^{-1}\ min^{-1}$.

The concentration dependencies of $k_{obs,i}$ and $k_{obs,ii}$ (Fig. 3) observed

through S could reflect two independent binding sequences, corresponding to

$$A + Ca^{2+} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} C \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} D \qquad \text{with} \qquad K_a = k_a/k_{-a} \tag{1}$$

This represents fast binding of Ca^{2+} (step a), followed by a slower isomerisation (step b). However, the data in e.g. Fig. 3 (inset) suggest that two such schemes cannot be independent. This, together with literature data (4,13) can require an additional equilibrium between two different protomers of Ni^{2+}-con A (P_1 and P_2) :

$$\begin{array}{ccccccc} P_1 + Ca^{2+} & \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} & (CaP_1)' & \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} & CaP_1 & \xrightarrow{i} & CaP_1S \\ k_{+5} \uparrow\downarrow k_{-5} & & & & & \overset{+S}{\curvearrowright} & \\ P_2 + Ca^{2+} & \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} & (CaP_2)' & \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} & CaP_2 & \xrightarrow[ii]{} & CaP_2S \end{array} \tag{2}$$

provided that this step (5) is faster than isomerisations 2 and 4. $(CaP_1)'$, CaP_1, $(CaP_2)'$, and CaP_2 are different forms of Ni^{2+}-Ca^{2+}-con A; CaP_1S and CaP_2S can be identical and represent the carbohydrate-protein complex with complete S-fluorescence quenching (5). Fast binding of Ca^{2+} in steps 1 and 3 is followed by slower isomerisations in steps 2 and 4. The inset of Fig. 3 suggests that solutions of stable Ni^{2+}-con A contain mostly the slowly reacting P_2 form. However the amount of CaP_1S formed <u>via</u> P_1 in the faster process i increases with $[Ca^{2+}]$ at the expense of P_2 and $(CaP_2)'$, and at the expense of CaP_2S formed in the slower process ii. Scheme (2) can also apply to the kinetics of Ca^{2+}-induced absorption decrease if one assumes a Tyr absorption decrease in step 4, but no change in step 2. The very fast absorption decrease, occuring in less than 5 sec, is attributed to a Ca^{2+}-induced shift in equilibrium 5 of P_2 towards P_1 with the lower Tyr absorption. Increasing $[Ca^{2+}]$ increases the fraction of Ca^{2+}-binding <u>via</u> steps 1 and 2, which are unobserved in Tyr absorption, and shifts P_2 to P_1, causing the increase of the fractional contribution in the sec-time range.

This concentration dependence of the biphasic or monophasic Ca^{2+}-binding kinetics observed in the min-time range at 16 °C can be related

with a temperature dependent equilibrium (4) between two forms of Ni^{2+}-con A in solution as postulated for Mn^{2+}-con A (4) and apo-con A (13). Such two different forms of Ni^{2+}-con A protomers, in which we assume differences in Tyr absorbance, have been demonstrated in the asymmetric dimeric Mn^{2+}-con A (18) and in apo-con A (19). The latter dimer shows differences in the metal binding region and in the positions of Tyr 12 and Tyr 100. These have also been invoked in CD studies on Ca^{2+} binding to concanavalin A (1).

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